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Molecular monitoring and characterization of the faecal microbiota of healthy dogs during fructan supplementation

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Abstract

The large intestine of dogs contains a complex microbial ecosystem with predominance of streptococci, bifidobacteria, lactobacilli, *Bacteroides* and *Clostridium*. Generally, this predominant microbiota in dogs is relatively stable in time but much less is known about its taxonomic composition. Moreover, almost no studies have been conducted to investigate this stability of the faecal microbial population in dogs upon prebiotic administration. The objective of the present study was to monitor possible changes in faecal microbiota of seven healthy adult dogs related to the administration of two fructans, oligofructose and inulin. For this purpose, population fingerprints generated by denaturing gradient gel electrophoresis (DGGE) analysis of universal V3 16 S rRNA gene PCR amplicons were compared between control (baseline) samples and samples collected after prebiotic feeding. From these DGGE gels, marked changes were observed in the faecal microbiota between subjects and before and after fructan administration. One DGGE band that appeared or intensified after fructan intake was further analyzed. Sequence analysis could attribute this band to a member of the *Streptococcus bovis–equinus* group. Following cultivation on MRS medium, a set of faecal isolates that most likely represent the stimulated streptococci were allocated to the species *Streptococcus lutetiensis* by $(GTG)_5$-PCR fingerprinting and partial 16 S rRNA and *sodA* gene sequencing. The data provided in this study demonstrate the ability of fructans to influence the bacterial composition of the gut microbiota in healthy dogs. More work is needed to unravel the relevance of *S. lutetiensis* or other autochthonous organisms of the dog gut as target groups for prebiotic supplementation.

Keywords: Fructans; Faecal microbiota; DGGE, Dogs; *Streptococcus lutetiensis*

1. Introduction

Like most mammals, the large intestine of healthy dogs contains a complex microbial ecosystem in which streptococci, bifidobacteria, lactobacilli, *Bacteroides* and *Clostridium* constitute the main predominant bacterial genera [1,2]. The large number of bacteria (i.e., 10^{10} per g dry faeces) [3] contributes significantly to the colonic fermentation of complex carbohydrates and proteins leading to the production of beneficial short chain fatty acids (SCFA) and several toxic putrefactive components (ammonia, indoles, phenols), respectively. The relative ratio in which these components are produced is highly dependent on the bacterial balance and substrate availability in the colon [4]. Not much is known about the exact composition of the microbiota of dogs and generally information at the species level is lacking.

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Substrate availability has been shown to have an impact on the production of fermentation end-products. Non-digestible carbohydrates such as the fructans oligofructose and inulin are prebiotic substrates that can selectively stimulate beneficial endogenous gut bacteria such as lactic acid bacteria and *Eubacterium*, thereby improving host health. Because the fructose units of oligofructose (2–8) and inulin (2–60) are connected by β-(2,1) linkages [5], these components are resistant to mammalian digestive enzymes [6] which makes them available for colonic bacteria. In conjunction with prebiotic-induced bacterial population shifts, several other effects associated with prebiotic ingestion have been reported including increased SCFA and vitamin synthesis, decreased production of putrefactive components and stimulation of gastro-intestinal physiology, immune function, lipid metabolism and nutrient digestibility [7–9]. However, most of these claimed health benefits are demonstrated in humans whereas there are few data available on prebiotic effects in domesticated animals such as dogs.

The vast majority of the prebiotic feeding studies involving dogs are based on the analysis of physiological effects, nutrient digestibility, stool quality and faecal concentrations of fermentative end products [10–12]. Prebiotic induced changes of the composition of the intestinal microbiota in dogs have mainly been studied by conventional plate counting methods [13] which lack the sensitivity to fully reflect bacterial diversity in faecal samples from dogs in comparison with molecular approaches such as PCR amplification or fluorescent in situ hybridization (FISH) [14–16]. As a follow-up to a previous prebiotic feeding experiment [11], it was the aim of the present study to analyze possible population changes associated with oligofructose and inulin administration within the predominant faecal microbiota of healthy adult dogs by culture-independent monitoring [17,18]. For this purpose, population profiles generated with denaturing gradient gel electrophoresis (DGGE) [17] of 16S rRNA gene V3 and V6–V8 amplicons were compared between control samples and samples collected upon fructan supplementation. One of the changes that was revealed by DGGE analysis in the predominant microbial gut population was further elaborated polyphasically by DGGE band sequence analysis, bacterial isolation and molecular identification.

2. Materials and methods

2.1. Faecal samples and total DNA extraction

Dog faecal samples originated from a previous study in which the effects of oligofructose and inulin administration on nutrient intake and digestibility, stool quality and faecal protein catabolites were evaluated [11]. From each of the seven dogs included in the latter study (designated C, D, G, M, O, S and T), three faecal samples were subjected to DGGE analysis: a control sample obtained before fructan administration and two composite samples obtained during the four day collection period after 10 days administration of oligofructose (4.5 g/day) and inulin (5.6 g/day), respectively. Surgical and animal care procedures were approved by the Institutional Animal Care and Use Committee, University of Illinois at Urbana-Champaign. Samples were stored at −20 °C.

Faecal samples were homogenized and total DNA extraction was performed as previously described [19].

2.2. PCR amplification and DGGE

Amplification of the V3 and the V6–V8 region of the 16S rRNA gene was performed using the primer pairs F357-GC and R518, and U968-GC and L1401, respectively. DGGE analysis of the V3 PCR amplicons was performed using a 35–70% denaturing gel, whereas a 40–60% denaturing gradient was used for the separation of V6–V8 PCR amplicons. DGGE gels were stained with 1× SYBR® Gold (catalog no. S-11494, Molecular Probes) for 30 min and further normalized and analyzed using the BioNumerics software, version 3.50 (Applied Maths, St.-Martens-Latem, Belgium). The same procedure was followed as described previously [19].

2.3. DGGE band sequencing

After photographing the gel, selected DGGE bands were cut out of the gel with a sterile scalpel followed by elution of the DNA by adding 50 μl TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and incubation overnight at 4 °C. The resulting DNA extract was subjected to V3-16S rRNA gene PCR after which PCR products were analyzed by DGGE together with the amplicons from the original samples in order to verify that the correct bands were isolated. Upon confirmation, a second round of PCR was carried out with non-GC-clamp primers. Following purification of the PCR amplicons using the QIAquick PCR Purification Kit (Qiagen), sequencing was performed using the Big Dye™ Termination RR Mix V3.1 (Applied BioSystems) on an ABI 3100 automated DNA sequencer (Applied BioSystems). For each sequencing reaction, a 10 μl reaction mixture was prepared, consisting of 0.67 μl Big Dye™, 1.66 μl 5× sequencing buffer (Applied BioSystems), 3 μl V3 forward primer without GC clamp or V3 reverse primer (5 μM), 3.67 μl sterile Milli-Q water and 1 μl of the purified PCR product. The PCR profile consisted of 30 cycles of denaturation at 96 °C for 15 s, annealing at 35 °C for 1 s and extension at 60 °C

for 4 min followed by cooling to 20 °C. The PCR products were purified by adding 25 μl absolute ethanol and 1 μl 3 M Na-acetate (pH 4.6). After cooling on ice for 10 min, samples were centrifuged at 20,000*g* for 25 min. The pellet was washed with 250 μl ethanol (70%) and vacuum dried for 25 min. Sequence assembly was performed using the AutoAssembler program (Applied Biosystems). The closest related sequences were found using the fasta program (http://www.ebi.ac.uk/fasta33/index.html).

2.4. Bacterial isolation

From a selection of the fructan-treated samples (C3, D3, G3, M3, O3, S3 and T2), 10-fold dilution series (10^{-1}–10^{-7}) were prepared by suspending 1 g wet weight faeces in 9 ml peptone-physiological solution [PPS, 0.1% (w/v) Peptone (Oxoid, L37) and 0.85% (w/v) NaCl in distilled water]. A total of 50 μl of each dilution was plated on MRS agar (Oxoid) and incubated at 37 °C under micro-aerobic conditions. After incubation for 48 h, five colonies were selected from the highest dilution with growth for each sample and further purified on MRS agar (Oxoid).

2.5. Identification and typing techniques

A selection of the purified isolates was subjected to repetitive DNA element (rep)-PCR fingerprinting using the $(GTG)_5$ primer [$(GTG)_5$-PCR], Fluorescent Amplified Fragment Length Polymorphism (FAFLP) and partial sequencing of 16S rRNA and *sodA* genes. $(GTG)_5$-PCR was carried out using the $(GTG)_5$ primer 5′-GTG GTG GTG GTG GTG-3′ [20] as previously described [21]. FAFLP analysis was carried out as described by Masco et al. [22], using primers E00 (5′-GAC TGC GTA CCA ATT C-3′) and T00 (5′-CGA TGA GTC CTG ACC GA-3′) for the preselective PCR and primers E01-6FAM (5′-6FAM-GAC TGC GTA CCA ATT CA-3′) and T01 (5′-CGA TGA GTC CTG ACC GAA-3′) for the selective PCR. For partial 16S rRNA gene sequencing, an amplicon of 1533 bp obtained with the conserved primers pA (5′-AGA GTT TGA TCC TGG CTC AG-3′) and pH (5′-AAG GAG GTG ATC CAG CCG CA-3′) was purified with the QIAquick PCR Purification Kit (QIAgen) and partially sequenced (±1000 bp) using the primers *Gamma (5′-CTC CTA CGG GAG GCA GCA GT-3′) and pD (5′-GTA TTA CCG CGG CTG CTG-3′) according to the protocol described for DGGE band sequencing. For partial *sodA* sequencing, a 435-bp internal fragment was sequenced using the PCR program and the primers d1 and d2 described by Poyart et al. [23].

Partial 16S rRNA and *sodA* gene sequence data of isolates S3a and O3a were deposited in the EMBL nucleotide sequence database under Accession Nos. AJ843133–AJ843136.

3. Results

3.1. Faecal population profiling with DGGE

PCR-DGGE analysis with universal primers targeting the V3 or V6–V8 region of the 16S rRNA gene was used to study possible changes in the predominant faecal bacterial population of control samples and oligofructose and inulin treated samples. Overall, DGGE profiles of V3 and V6–V8 16S rRNA gene amplicons displayed a relatively low complexity. In contrast to the V6–V8 profiles (data not shown) that were very homogenous within a given subject, the V3 profiles tended to display more qualitative (presence or absence) or quantitative (intensity of bands) variations within each of the seven subjects tested (Fig. 1). Most of the observed changes appeared to be subject-specific. In addition, in multiple subjects a number of bands appeared or intensified in the control and/or in the oligofructose and inulin fed groups. One particular band fragment after

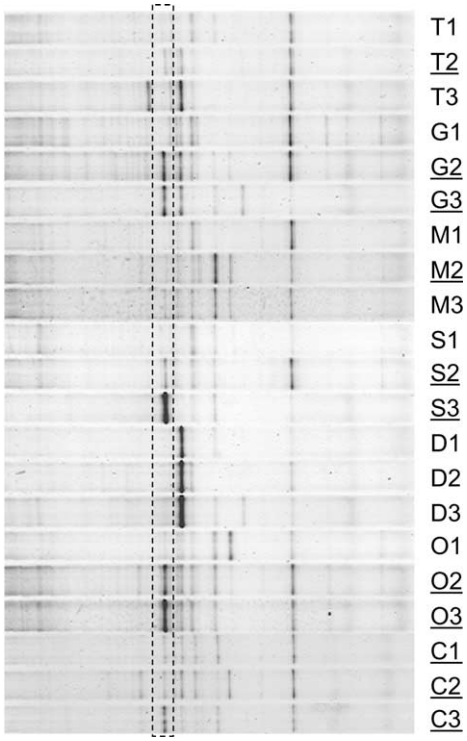

Fig. 1. Digitized DGGE profiles obtained with the universal V3 16S rRNA gene primer of the seven dog subjects (C, D, G, M, O, S, and T). Numbers in the sample codes refer to the control samples (1), samples obtained after oligofructose supplementation (2) and after inulin supplementation (3). The square contains an appearing or intensifying band after prebiotic administration. Underlined sample codes refer to the DGGE patterns from which the indicated band was extracted for sequence analysis.

oligofructose and/or inulin administration at the same position in the V3 profiles of all seven subjects except that of subject D. This change in the predominant bacterial population was most pronounced in subjects G, M, O, S and T where the specific band fragment was present in one or both of the samples obtained after feeding and could not be detected in the respective control samples. In subject C, this band fragment was detected in both the control sample and in the samples obtained after fructan supplementation whereas it remained undetected in all samples of subject D (Fig. 1).

3.2. DGGE band sequence analysis

In order to determine to which bacterial group or taxon this particular band could be ascribed, the specific DGGE band fragment that appeared or intensified after feeding was further characterized by sequencing analysis. This band was sampled from 11 V3 profiles (Fig. 1) and yielded identical 170 bp sequences in all samples. After comparison with the EMBL database, the sequence of the band in question exhibited highest similarity (99.42%) with the 16S rRNA gene sequence of species belonging to the *Streptococcus bovis–equinus* group. However, because of the short length of the sequenced V3 fragment (170 bp) and the very high V3-16S rRNA gene sequence similarity among members of the *S. bovis–equinus* group, the obtained sequence information did not allow to assign the 170-bp band to one specific *Streptococcus* species.

3.3. Isolation and identification of the predominant faecal streptococci

Given the dominance of the unidentified streptococcal group in the DGGE population fingerprints, an attempt was made to isolate representatives of this group from a selection of faecal samples (C3, D3, G3, M3, O3, S3 and T2) on MRS medium under micro-aerophilic conditions favouring the recovery of streptococci. In this way, a maximum of five colonies (designated a to e) per sample were selected and further purified for PCR-DGGE analysis using the V3-16S rRNA gene primer. By comparing DGGE band positions, it was found that four out of the seven investigated samples (i.e., C3, O3, S3 and T2) contained several isolates that exactly matched the streptococcal group (Fig. 2). Subsequently, these isolates were further analyzed with $(GTG)_5$-PCR fingerprinting and compared with type and reference strains of species belonging to the *S. bovis–equinus* group including *Streptococcus alactolyticus*, *S. bovis*, *S. equinus*, *Streptococcus gallolyticus* subsp. *gallolyticus* and subsp. *macedonicus*, *Streptococcus lutetiensis* and *Streptococcus infantarius* subsp. *infantarius* (Fig. 3).

Clustering analysis of $(GTG)_5$-PCR profiles indicated that the faecal isolates were most closely related to *S. lutetiensis*. Numerical analysis of fingerprints also showed that two $(GTG)_5$-PCR types, i.e., type 1 and 2, were common to dogs C, O, S and T and dogs C, S and T, respectively. Both strain types differed in a single band-position and all members within each of these types displayed highly similar if not identical banding patterns. Per dog, one representative isolate of each $(GTG)_5$-PCR type was further studied by FAFLP fingerprinting. Numerical analysis and visual inspection of the FAFLP patterns showed that all of the selected isolates (S3b, C3c, T2e, O3a and T2d, C3d, S3a) displayed similar but individually distinct profiles (data not shown).

The partial 16S rRNA gene sequences of isolates O3a (representing $(GTG)_5$-PCR type 1) and S3a (representing $(GTG)_5$-PCR type 2) showed 99.9–100% similarity with three sequence entries of *S. lutetiensis* (EMBL Accession. Nos. AF429763, AJ297214 and AJ297215) including that of the type strain (CIP 106849^T). However, a very high 16S rRNA gene sequence similarity

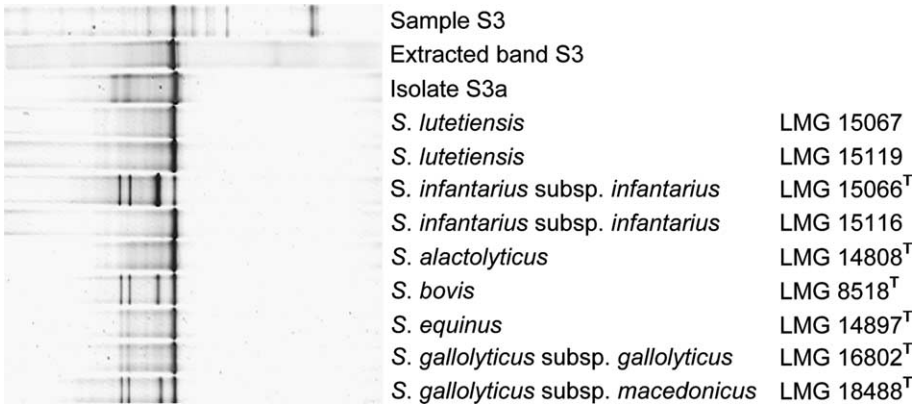

Fig. 2. Comparison of V3 16S rRNA gene primer DGGE profile of sample S3 with the DGGE band positions of the extracted band of sample S3, isolate S3a, and type and reference strains of the *Streptococcus bovis–equinus* group. Numbers behind the species assignment indicate the BCCM-LMG strain numbers. T, type strain.

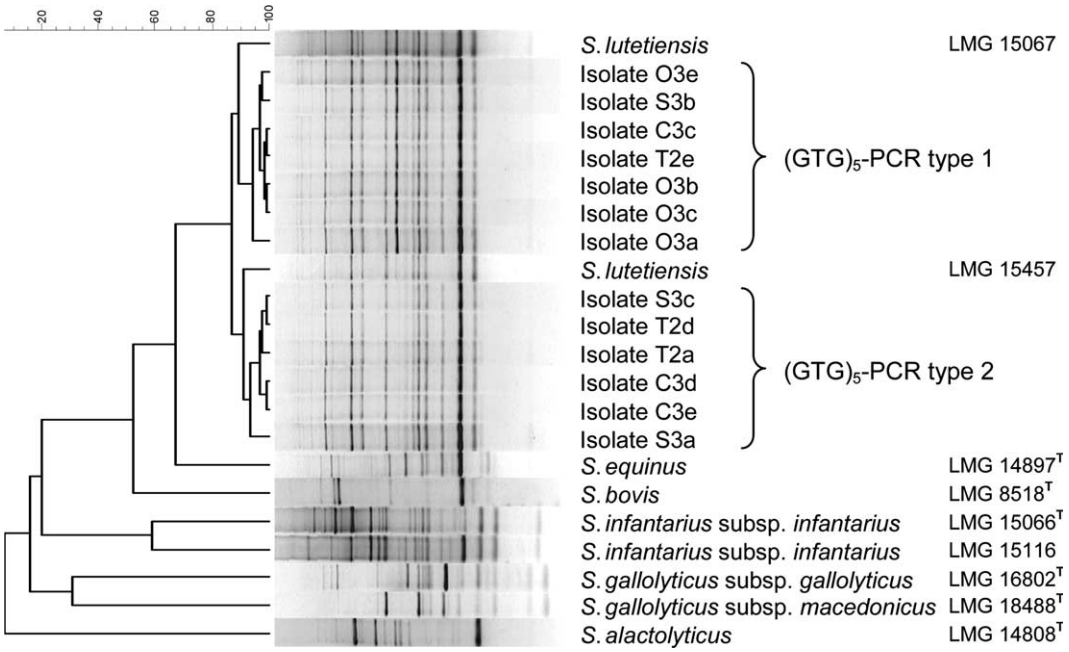

Fig. 3. Clustering analysis of $(GTG)_5$-PCR profiles of a selection of faecal isolates and type and reference strains of *S. bovis–equinus* group members using the Pearson correlation coefficient and the UPGMA method.

was also found between the two dog isolates and other members of the *S. bovis–equinus* group such as *S. infantarius* subsp. *infantarius* (99.7%) and *S. equinus* (99.5%), which makes it impossible to reliably assign these isolates to a specific streptococcal (sub)species. In contrast, both isolates showed a partial *sodA* sequence similarity ranging from 99.8% to 100% with the *sodA* gene of *S. lutetiensis* strains (EMBL Accession Nos. AY035713, AJ297188 and AJ297205) but only displaying 89.4–90.3% similarity with *sodA* sequences of *S. bovis*, *S. equinus* and *S. infantarius* subsp. *infantarius*.

4. Discussion

Among various beneficial effects associated with prebiotic administration in dogs [10–12], the decrease of faecal odor components implicated in carcinogenesis has received much attention in recent years. Several dose–response experiments conducted in dogs with oligofructose and inulin have shown beneficial effects towards an improved small intestine absorption capacity and colonic epithelial cell proliferation [24], whereas future trends in prebiotic dog feeding aim at the controlled decrease of faecal putrefactive components and at an improved nutrient digestibility. In a previous prebiotic feeding study in seven healthy adult dogs [11], supplementation of fructans resulted in an overall increase in faecal SCFA concentrations and decrease in total phenols. However, the possible link between the observed fructan-induced effects and specific changes or shifts in the dog microbiota so far remain unclear. The objective of the current study was to monitor overall changes in the predominant faecal microbiota of these seven dogs after oligofructose or inulin administration and identify the bacterial group(s) involved in one of the most pronounced population changes. For this purpose, the diversity and stability of the predominant faecal bacterial population were analyzed by comparing PCR-DGGE fingerprints of the control faecal samples with those of samples collected after prebiotic feeding. In this comparison, it was assumed that the predominant microbiota of dogs is relatively stable in time [25] as has also been observed in humans [19] and pigs [26]. Despite their low complexity, V3 PCR-DGGE profiles showed considerable variation between subjects indicating that each of the dogs in the study harbors a unique faecal population. Next to subject-specific changes, the appearance or intensification of several bands resulting from fructan administration could be observed. One specific V3 band fragment obtained after feeding was observed in six out of the seven subjects and was selected for further characterization. In contrast, Simpson et al. [25] reported that DGGE population fingerprints remained relatively stable upon dietary fiber supplementation in dogs. However, as noted by the latter authors, both the concentration and the type of prebiotic substrate may significantly influence its effect at the microbial population level.

Sequence analysis of the extracted and purified V3 amplicon revealed highest homology with members of the *S. bovis–equinus* group. Based on this information,

streptococci could be isolated from faecal samples that produced V3 amplicons at the same position in DGGE profiling as that of the original sample amplicon. Polyphasic taxonomic characterization with $(GTG)_5$-PCR fingerprinting and partial 16S rRNA and *sodA* gene sequencing allocated these faecal isolates to the species *S. lutetiensis*. The V3 16S rRNA gene sequences of the *S. lutetiensis* isolates were 100% identical to the sequence of the extracted band fragment (data not shown). Taken together with the relative dominance of this band in the DGGE profiles of the samples obtained after feeding (Fig. 1), it is thus very likely that the faecal *S. lutetiensis* isolates represent the streptococcal group that appeared to be involved in one of the most pronounced population changes observed after fructan administration. Although these isolates originated from different dogs, $(GTG)_5$-PCR revealed that three subjects shared two *S. lutetiensis* strain types that differed only in a single band position. FAFLP fingerprinting, which is considered to be the most discriminating typing method next to pulsed-field gel electrophoresis, unraveled that members of these two $(GTG)_5$-PCR types are highly related but most probably represent different strains.

Although bifidobacteria are generally considered to be one of the main target groups during fructan supplementation in mammalians, none could be detected in the present study using a *Bifidobacterium* genus-specific PCR (data not shown). Willard et al. [27] could only sporadically isolate bifidobacteria from dog faeces whereas several other studies [13,28,29] reported an increase of maximum one log unit. However, it should be noted that all these studies relied on conventional plate counting methods which often lack specificity for bifidobacterial enumeration in faecal samples.

Although streptococci constitute one of the most predominant bacterial groups in the faecal microbiota of healthy dogs [1,2,16], few studies have examined their taxonomic diversity in canine gut flora. A recent study has indicated that *Streptococcus alactolyticus* is the dominant culturable species of lactic acid bacteria in the jejunum and faeces of fistulated dogs [30]. To our knowledge, the present study is the first to report the presence of *S. lutetiensis* in dogs. So far, this species has only been isolated from human clinical (blood and urine) and non-clinical (faeces) specimens [31,32]. Because of the limited knowledge regarding the distribution of *S. lutetiensis* (formerly named *S. infantarius* subsp. *coli* [33]), its ecological role in the gut still remains unclear. By definition, prebiotics are considered to selectively stimulate growth and/or activity of potentially health-enhancing intestinal bacteria [34]. However, at this stage, there are no indications to claim that a potential stimulation of *S. lutetiensis* by fructan administration exerts positive or negative effects on the health of the dogs. Clearly, additional research is needed to unravel the ecology of this organism in the canine gut and to investigate its role in the gut of healthy dogs. The possible effect of prebiotic fructans on autochthonous bacterial species of the dog gut such as *S. lutetiensis* requires further investigation in future studies including higher numbers of subjects. In addition, such studies should also focus on other potential target organisms for prebiotic substrates and would benefit from the use of quantitative culture-independent techniques such as real-time PCR and fluorescent in situ hybridization assays. These new insights are crucial to understand which microbe-host interactions are affected in dose–response feeding trials and may stimulate the development of more effective prebiotic formulations for dog health.

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